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## Decreased Hypoxia-Induced Neovascularization in Angiopoietin-2 Heterozygous Knockout Mouse through Reduced MMP Activity

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### Key Words

Angiopoietin-2 • Diabetic retinopathy • Angiogenesis • Matrix metalloproteinase

### Abstract

**Background/Aims:** Proliferative diabetic retinopathy is characterized by the formation of retinal neovascularization. Angiopoietin-2 (Ang-2) and matrix metalloproteinase (MMP) play a critical role in angiogenesis. However, the precise location and function of Ang-2 during formation of retinal neovascularizations driven by hypoxia in relation to MMP activity have not been elucidated. In this study, we investigated the response of Ang-2 heterozygous knockout retinas (Ang2<sup>+/-</sup> mouse) to hypoxia and its link to MMP activity in an oxygen-induced retinopathy (OIR) model. **Methods:** Pre-retinal neovascularizations were quantitated in vertical sections. Intra-retinal angiogenesis was assessed by whole mount immunofluorescence staining of retinas. MMP activity was examined in retinal protein lysate and whole mount retinal in situ zymography. **Results:** Ang2<sup>+/-</sup> retinas subjected to the OIR model showed 33 % reduced neovascularization and 271 % increased avascular zones at postnatal day 17. In the OIR model, Ang-2

was modestly expressed in pre-retinal neovascularizations and venules, but strongly in arterioles and capillary sprouts. MMPs were activated in close association to where Ang-2 is expressed. MMP activity was substantially decreased in Ang2<sup>+/-</sup> retinas. **Conclusions:** Our present data suggest the spatially concomitant expression of Ang2 and MMPs, and that Ang2 modulates hypoxia-induced neovascularization by regulating MMP activity.

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### Introduction

Proliferative diabetic retinopathy is still the leading cause of blindness in the working age all over the world [1]. There is emerging evidence indicating that the Angiopoietin-Tie2 system is involved in the pathogenesis of diabetic retinopathy [2, 3]. Hyperglycemia up-regulates angiopoietin-2 (Ang-2) expression prior to the morphological changes of retinal capillaries in diabetic retinopathy. Pericyte loss is causally linked to elevated Ang-2 level in the retina. Pericytes are important for the protec-

tion of endothelial cells against capillary damage. Loss of pericytes is followed by degeneration of endothelial cells and capillary occlusion [4]. Regression of retinal capillaries and insufficient oxygen supply lead to hypoxia-induced lesions of the retina. Hypoxia-induced neovascularization is the hallmark of proliferative diabetic retinopathy (PDR). The newly formed blood vessels grow on the inner limiting membrane of the retina towards the vitreous. They are thin-walled and leaky, leading to exudates and haemorrhages, causing severe vision loss and even blindness in end stages of the disease. Ang-2 is elevated in vitreous fluid and epi-retinal membranes of patients with PDR [5, 6]. Furthermore, Ang-2 is upregulated in experimental rodent retinas with hypoxia-induced neovascularization (OIR) [7]. Vascular endothelial growth factor (VEGF) is a major mediator in the hypoxia-induced neovascularization [8, 9]. Expression of VEGF in concert with Ang-2 under hypoxia induces retinal neovascularizations [10, 11].

The mechanisms underlying Ang-2-involved retinal neovascularization in proliferative diabetic retinopathy are complex. The most important initial step in retinal neovascularization is the degradation of the basement membrane and extracellular matrix components surrounding the endothelial cells followed by invasion, migration and proliferation of endothelial cells and final formation of new vascular tubes [12]. Matrix metalloproteinases (MMPs) are among the most potent regulators of angiogenesis during extracellular matrix remodelling. MMP-2 and MMP-9 are upregulated in vitreous and epi-retinal membrane of patients with PDR [13-15]. MMP activity is increased in experimental rodent retinas of hypoxia-induced neovascularization [16]. Reduction of retinal neovascularization in MMP-2 and in MMP-9 null mice and by MMP inhibitors suggests the importance of such proteases in retinal neovascularization [13, 16]. Angiogenic stimuli, such as VEGF and Ang-2, promote the expression of MMPs [17, 18]. In tumor tissues, upregulation of MMPs in the endothelial cells by Ang-2 may contribute to cell invasion [19]. MMPs colocalize with Ang-2 in the invasive areas of glioma tissues, indicating the synergistic effect of MMPs and Ang-2 in tumor cell invasion [20, 21]. Furthermore, a study by Das et al. suggested that Ang-2 stimulates MMP expression in cultured endothelial cells in a dose dependent manner, and blocking of the Ang-Tie2 system by soluble Tie-2, a common receptor for Ang-1 and its antagonist Ang-2 led to decreased hypoxia-induced neovascularization and MMP-9 expression in the OIR model [18].

Although Ang-2 and MMPs are detected in the

neovascularization areas in OIR [18, 22], little is known about the precise requirement of Ang2 and MMP activity in OIR. Furthermore, no detailed information is available on the MMP activity related to decreased Ang-2 in OIR. Using a Ang-2 heterozygous knockout mouse (Ang2<sup>+/-</sup> mouse) in the OIR model, we investigated the vascular response of Ang2<sup>+/-</sup> retinas to hypoxia, the expression of Ang-2 and activity of MMP under hypoxia in Ang2<sup>+/-</sup> mouse using the reporter gene LacZ and whole mount retinal in situ zymography, respectively.

## Materials and Methods

### *Animals*

The care and experimental use of all animals in the study were in accordance with institutional guidelines and in compliance with the Association for Research in Vision and Ophthalmology (ARVO) statement. Genotyping was performed as described previously [23].

### *Analysis of retinal neovascularization in the OIR model*

To study the vascular response of Ang2<sup>+/-</sup> retinas to hypoxia, newborn Ang2<sup>+/-</sup> mice were subjected to 75 % oxygen at postnatal day 7 (p7) for 5 days with their wild type littermates and nursing mothers. Hyperoxia results in vasoregression in the superficial capillary layer, while the deep capillary layer does not exist at this time point. At p12, the mice were returned to room air. Relative hypoxia from p12 to p17 leads to pre-retinal neovascularization and intra-retinal vascular generation/repair in the retinal superficial and deep capillary layer. At p17, eyes were enucleated under deep anaesthesia. For neovascularization analysis, eyes were immediately fixed in formalin, then, embedded in paraffin. Serial sections of 6 µm thickness were generated and the numbers of pre-retinal neovascular nuclei per section were counted as described previously [24].

### *LacZ staining*

To assess the expression of Ang-2 in hypoxia, we used the reporter gene LacZ in this mouse. Eyes were obtained from Ang2<sup>+/-</sup> and their wild type littermates at p12 and p17 in OIR model. They were first fixed with 0.2 % glutaraldehyde and 1.5 % formaldehyde in 100 mM phosphate buffer pH 7.3 for 20 minutes, then, retinas were enucleated. After washing, the retinas were stained in a solution containing 0.1 % X-gal (Roche Diagnostics GmbH, Mannheim, Germany), 5 mM K<sub>4</sub>Fe(CN)<sub>6</sub>, and 5 mM K<sub>3</sub>Fe(CN)<sub>6</sub> at 37°C for 6 hours. Finally, retinas were washed in PBS and subjected to immunofluorescence staining.

### *Whole mount vessel immunofluorescence and quantification of avascular zones*

To evaluate avascular zones resulting from hyperoxia at p12 and its alteration due to relative hypoxia at p17 in the OIR model, eyes subjected to LacZ staining were subsequently

stained with FITC-conjugated lectin (1:100, Sigma, Germany) to visualize retinal vessels. Central avascular zones in the retina (measured in  $\mu\text{m}^2$  per retina) were quantified by using an analysis program (IM50, Leica BMR, Bensheim, Germany). To identify the colocalization of Ang-2 with pericytes, double staining was performed. Vessels were recognized by TRITC-conjugated lectin (1:100, Sigma, Germany) and pericytes were identified by pericyte-specific marker rabbit anti-NG2 (1:200, Chemicon International, Hampshire, UK) as first antibody and swine anti-rabbit IgG conjugated with FITC as secondary antibody (1:20, DakoCytomation GmbH, Hamburg, Germany).

#### *Retinal digest preparation*

Retinas of Ang2<sup>+/-</sup> mice from OIR model at p17 stained with LacZ were fixed in 4 % paraformaldehyde for 24-48 hours. Then, retinas were incubated in aqua bidest for 30 minutes. Subsequently, the retinas were transferred to a 3 % trypsin solution resolved in 0.2 M Tris buffer, pH 7.4, for 2-3 hours. During digestion, vitreous with pre-retinal neovascularizations of Ang2<sup>+/-</sup> retinas were carefully separated from intra-retinal vessels, flat mounted on objective slides and finally stained with PAS. The intra-retinal vasculature of Ang2<sup>+/-</sup> mice were also washed in aqua bidest. To visualize the vascular sprouts growing downwards to the deep capillary layer at p17 in the OIR model, photos were taken during the washing step with the focus on the deep capillary layer.

#### *MMP Activity - Gelatinase/Collagenase assay*

EnzChek Gelatinase/Collagenase Assay kit (Invitrogen GmbH, Karlsruhe, Germany) was used to detect MMP activities in the retinas. The kit contains DQ<sup>TM</sup> gelatine conjugated with fluorescein. This substrate can be efficiently digested by most gelatinases and collagenases to yield highly fluorescent peptides. Protein lysates were generated from OIR retinas at p17. 150  $\mu\text{g}$  proteins were incubated in a reaction buffer containing 50 mM Tris, 150 mM NaCl, 5 mM CaCl<sub>2</sub>, 0.2 mM sodium azide with 20  $\mu\text{g}$  DQ gelatine solution at room temperature. Fluorescence was measured at multiple time points in a fluorescence multi-well microplate reader set for excitation at 485 nm and emission at 530 nm. *Clostridium* collagenase and 1,10-phenanthroline were used for standard curve and MMP inhibitor, respectively. One unit is defined as the amount of enzyme required to liberate 1  $\mu\text{mole}$  of L-leucine equivalents from collagen in 5 hours at 37°C, pH 7.5.

#### *Detection of specificity of activated MMP*

20  $\mu\text{g}$  retinal proteins extracted from Ang2<sup>+/-</sup> p17 and WT p17 mice subjected to OIR were incubated with loading buffer without denaturation agents for 20 minutes on ice. The proteins were separated a 10 % SDS gel copolymerized with 0.1 % gelatin (Sigma-Aldrich, Taufkirchen, Germany). After electrophoresis, the gel was incubated in the zymogram renaturation buffer (Bio-Rad, Munich, Germany) for 1 h and followed by incubation in the zymogram development buffer (Bio-Rad, Munich, Germany) at 37°C for 48 hours. Subsequently, the gel was stained with 0.1 % coomassie blue R-250 (Serva, Heidelberg, Germany) containing 30 % methanol and 10 % acetic acid. Destaining was performed in a solution with 50 %

methanol and 10 % acetic acid. MMP activity was digitized as transparent bands with Gel IX imager system (INTAS, Göttingen, Germany).

#### *Whole mount retinal in situ zymography*

Detection of MMP activity was performed using EnzChek Gelatinase/collagenase Assay kit supplied by Molecular Probes (Invitrogen GmbH, Karlsruhe, Germany). Briefly, slides were pre-coated with 0.5 mg/ml DQ gelatine conjugated with fluorescein containing 0.1% agarose. Unfixed retinas of Ang2<sup>+/-</sup> and wild type mice subjected to OIR at p17 were isolated in cold PBS and whole mount mounted (ganglion cell layer downwards to coated slide). The preparations were then incubated in the reaction buffer at 37°C over night. Photos were taken with a fluorescence microscope connected to a digital camera (IM50, Leica BMR, Bensheim, Germany).

#### *Statistic analysis*

Data are expressed as mean  $\pm$  SEM. Data analysis was performed by using unpaired t-test with welsh correction. A p value < 0.05 was considered as statistical significant.

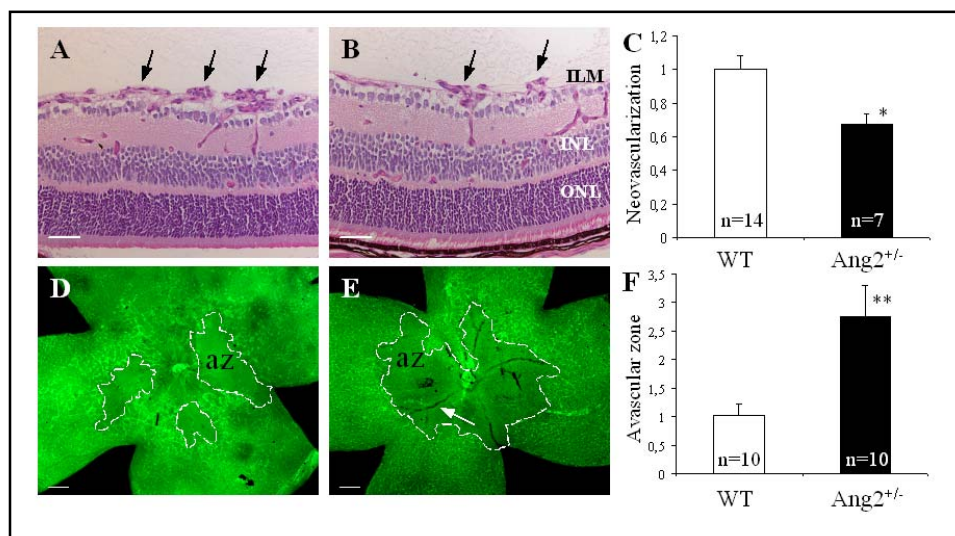
## **Results**

### *Decreased Ang-2 in the retina abrogates neovascular response of retinal vessels to hypoxia*

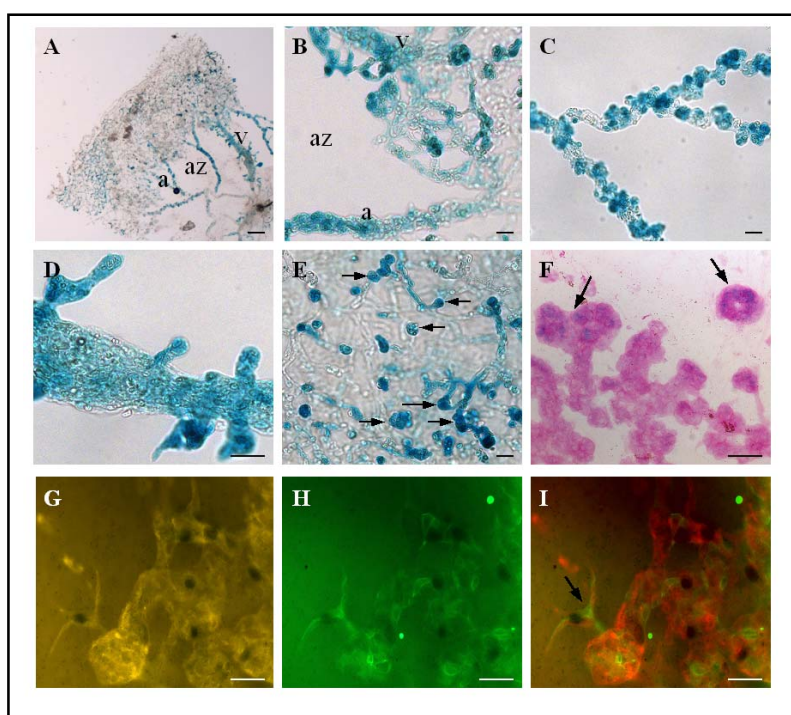
We used the OIR model to study the impact of Ang-2 on neovascularization in Ang2<sup>+/-</sup> mice at p17. First, pre-retinal neovascularizations were evaluated. Ang2<sup>+/-</sup> mice showed significantly reduced neovascularization in response to hypoxia at p17 ( $0.67 \pm 0.07$ ) compared with their wild type littermates ( $1.00 \pm 0.09$ ). The 50% reduction of Ang-2 gene dosage resulted in a 33% reduction of neovascularization in the retina (Fig. 1A-C). At p7 when the mice were subjected to the OIR model, Ang2<sup>+/-</sup> mice showed vascular development in the superficial layer identical to wild type mice (not shown).

Next, intra-retinal angiogenesis was assessed by measuring avascular zones in the retinas. Avascular zones resulting from hyperoxia in the OIR model were analysed at p17. Avascular zones at p17 differed significantly between Ang2<sup>+/-</sup> and wild type mice as depicted in Fig. 1D and E. Ang2<sup>+/-</sup> mice had a 271 % larger avascular area in the central retina compared with wild type mice (Fig. 1F). To determine whether decreased Ang-2 impairs the vasoregressive response in the OIR model, we measured the avascular zones at p12, immediately after the hyperoxic period when mice returned to room air. Retinas of Ang2<sup>+/-</sup> and wild type mice were comparable in the vascular zones at p12 ( $4.77 \pm 0.47 \text{ mm}^2/\text{retina}$  in Ang2<sup>+/-</sup> mice and  $4.82 \pm 0.60 \text{ mm}^2/\text{retina}$  in wild type

**Fig. 1.** Response of retinal vessels of Ang2<sup>+/-</sup> mice in oxygen-induced retinopathy mouse model. Representative demonstration (A and B) and quantification (C) of neovascularization in wild type (A) and Ang2<sup>+/-</sup> (B) mice at p17 in the OIR model. Arrows in A and B: pre-retinal neovascularizations. ILM: inner limiting membrane; INL: inner nuclear layer; ONL: outer nuclear layer. Whole mount retinas stained with lectin-FITC (D-E) showing avascular zone (az) in wild type (D) and Ang2<sup>+/-</sup> (E) mice at p17 in the OIR model. F is the quantitative analysis of avascular zones displayed in D and E. Arrow in E indicates arteriole stained positive for LacZ in Ang2<sup>+/-</sup> retinas. Values in wild type in each litter are normalized to 1. Data are expressed in mean ± SEM. \* p < 0.05, \*\* p < 0.01. WT: wild type mice; Ang2<sup>+/-</sup>: Ang2<sup>+/-</sup> mice. Scale bars: 50 µm (A and B), 200 µm (D and E).



**Fig. 2.** Expression of Ang-2 under hypoxia in oxygen-induced retinopathy mouse model. Representative demonstration of retinal digest preparations of Ang2<sup>+/-</sup> OIR retinas at p17 stained with LacZ. A shows an overview of ¼ retina with arteriole (a) venule (v) and avascular zone (az). Examples of vessels at border of avascular zone (B), arteriole (C) venule (D), sprouts towards the deep capillary layer (E) and pre-retinal neovascularizations (F) are displayed. F presents pre-retinal neovascularizations in the vitreous additionally stained with PAS. Photographs A - E were taken during washing step and E is focussed on the sprouts towards the deep capillary layer. G - H are double fluorescence analysis stained with lectin-Tritc (G) to visualize vessels and NG2 (H) to detect pericytes. Black points in G - H are LacZ-positive cells under fluorescence microscope. I: merged image of G and H. Arrows in E: sprouts towards the deep retinal layer. Arrows in F: LacZ weakly expressing pre-retinal tufts. Arrow in I: pericyte. n=5. Scale bars: 200 µm (A), 50 µm (B - I).

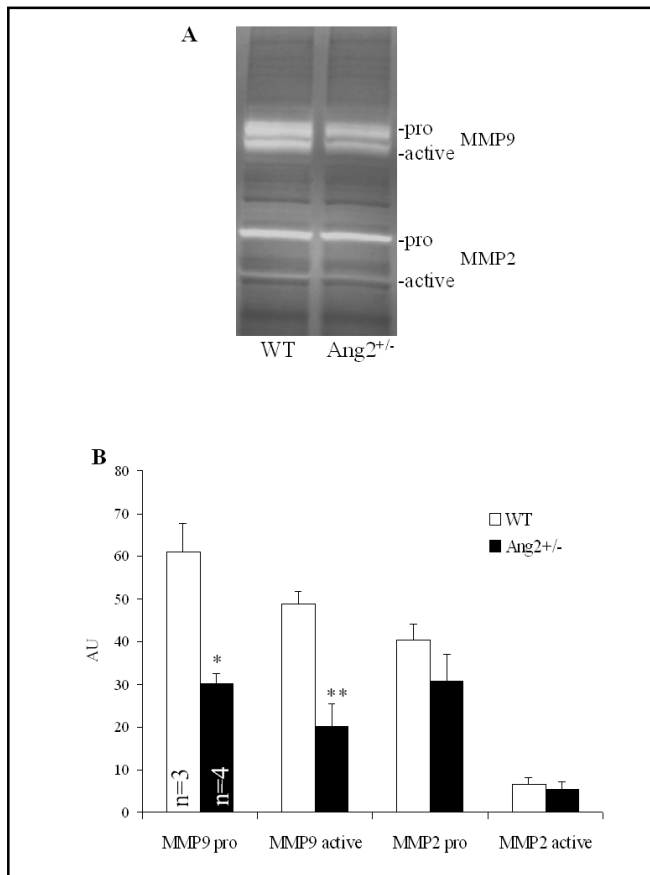


mice). The data suggested that Ang-2 plays a pivotal role in hypoxia-induced angiogenesis, but not in vasoregression under hyperoxia conditions in the OIR model.

To study the localization of Ang-2-expressing cells in retinal vasculature in the OIR model, we used the reporter gene in the Ang2<sup>+/-</sup> mice. As displayed in Fig. 2, Ang-2 was strongly expressed in vessels at p17 under retinal hypoxia. Ang-2 expression is detected in some capillaries in the periphery and in vessels at the border of

avascular zone in the retina (Fig. 2A and B), especially in tortuous arterioles (Fig. 2C), on sprouts of venules (Fig. 2D) and on sprouts towards the deep capillary layer (Fig. 2E). A scattered expression of Ang-2 was found in pre-retinal neovascular tufts (Fig. 2F). For colocalization of Ang-2 with vascular cells, immunofluorescence staining was performed in the Ang2<sup>+/-</sup> retinas at p17 in the OIR model. Lectin and NG2 were used to identify retinal vessels and pericytes, respectively, and Ang-2



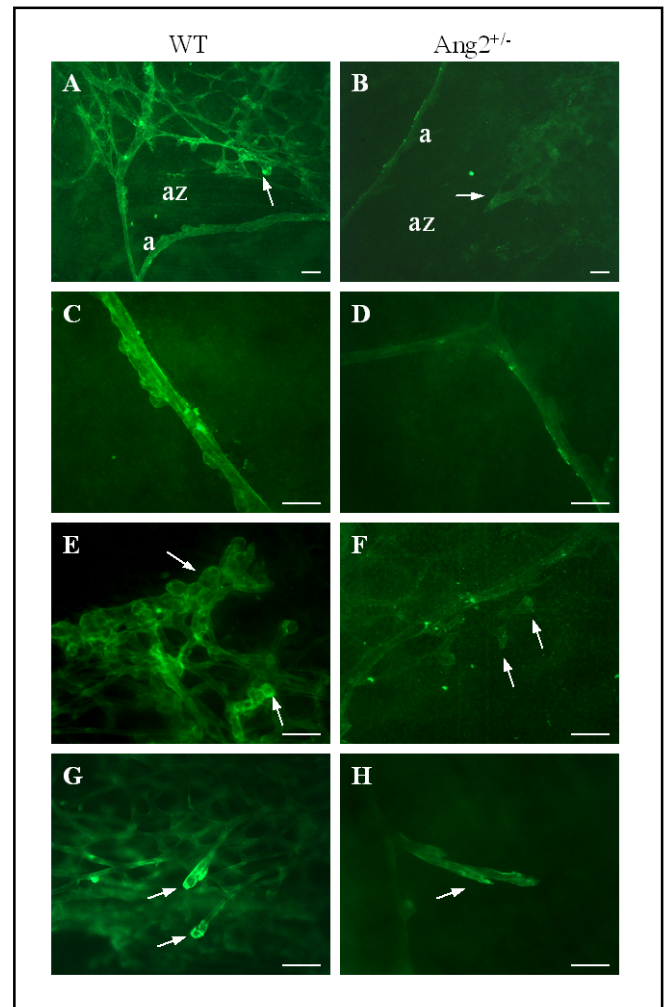


**Fig. 3.** Demonstration of MMP zymography gel of Ang2<sup>+/-</sup> retina at p17 in the OIR model. A: representative blots; B: Quantification of activity of MMP9 and MMP2. Ang2<sup>+/-</sup> retina shows decreased pro- and active-MMP9 compared with wild type retina. WT: wild type mice; Ang2<sup>+/-</sup>: Ang2<sup>+/-</sup> mice, AU: arbitrary units. \*  $p < 0.05$ , \*\*  $p < 0.01$ .

expression was determined by LacZ staining. As shown in Fig. 2G-I, expression of Ang-2 was detected in few neovascular tufts and on sprouts. Expression of Ang-2 was not colocalized with pericyte marker NG2, suggesting endothelial Ang-2 expression under hypoxia. Ang-2 was not detected in any vascular cell at p12 in the OIR model under hyperoxia (data not shown).

#### *Activity of MMP is reduced in the Ang2<sup>+/-</sup> retinas under hypoxia*

To examine the link between Ang-2 expression and MMP activity, we analysed the alteration of MMP activity in Ang2<sup>+/-</sup> retinas using gelatinase/collagenase assay with retinal total protein lysates and whole mount retinal in situ zymography. In the gelatinase/collagenase assay, Ang2<sup>+/-</sup> retinas at p17 in the OIR model showed signifi-



**Fig. 4.** Activity of MMPs in the Ang2<sup>+/-</sup> retinas at p17 in the OIR model. Ang2LacZ<sup>+/-</sup> retinas (B, D, F, H) show decreased MMP activity (green) compared with wild type retinas (A, C, E, G). A and B: the border of avascular zone, C and D: arterioles, E and F: pre-retinal neovascularization, G and H: capillary sprouts focused on the deep layer of the retina. az: avascular zone, a: arteriole. Arrows in A and B: capillary sprouts at the border of avascular zone. Arrows in E and F: neovascular tufts. Arrows in G and H: capillary sprouts towards deep capillary layer. The experiment was repeated twice. WT: wild type mice; Ang2<sup>+/-</sup>: Ang2<sup>+/-</sup> mice. Scale bars: 50  $\mu$ m.

cantly decreased MMP activity in the whole retinal protein lysates compared with wild type retinas (Ang2<sup>+/-</sup>:  $0.112 \pm 0.014$  mU/ $\mu$ g protein; wild type:  $0.171 \pm 0.013$  mU/ $\mu$ g protein,  $n=10$ ,  $p < 0.01$ ). MMP9 and MMP2 are the most abundant MMPs with gelatinase activity in the retina. Therefore, we further addressed the identity of MMPs regulated by hypoxia in Ang2<sup>+/-</sup> retinas. Both the pro- and active-MMP9 decreased significantly at p17

whereas pro- and active-MMP2 did not change in Ang2<sup>+/-</sup> retinas compared with wild type retinas subjected to the OIR model (Fig. 3). Furthermore, as displayed in Fig. 4, strongly activated MMPs were detected at the border of the avascular zone, i.e. where hypoxia-induced intra-retinal remodeling predominantly occurred in wild type mice. Arterioles and neovascularizations also demonstrated high MMP activity. Apart from capillary sprouts at the border of avascular zone, strong MMP activity was seen in the capillary sprouts extending towards the deep capillary layer. Activated MMPs in wild type retinas were exclusively found in areas in which Ang-2 was expressed (see corresponding Fig. 2). In Ang2LacZ<sup>+/-</sup> retinas in the OIR model, the activity of MMPs was substantially lower than in wild type retinas comparable with the results of the zymography performed on whole lysates. A modest staining intensity was observed in neovascularizations, in the vessels at the border of avascular zones, in arterioles and capillary sprouts towards the deep capillary layer, indicating a largely decreased activity of MMPs under reduction of Ang-2 in the OIR model.

## Discussion

Our study demonstrates that a reduction in Ang-2 gene dose results in reduced activity of MMP9 at the sites of angiogenic remodelling, and in decreased pre- and intra-retinal angiogenesis.

The main finding in this study is the concomitant spatial expression of Ang-2 and MMPs activity in the hypoxia-induced retinal neovascularization model, suggesting that Ang-2 and activated MMPs play a permissive role in sprouting angiogenesis. Furthermore, as the data in Ang2LacZ<sup>+/-</sup> mice indicate, decreased Ang-2 gene dose with consecutive reduced activity of MMPs leads to lower response of retinal vessels to hypoxia which implicates that Ang-2 exerts its effect through activation of MMPs in sprouting angiogenesis. Under hypoxia in OIR, reduction of Ang-2 leads to decreased intra-retinal angiogenesis indicated by increased avascular zone and decreased pre-retinal neovascularization. Reduced angiogenic response of Ang2<sup>+/-</sup> retinas in the OIR model demonstrates that angiogenesis induced by hypoxia-driven VEGF is modulated by Ang-2. Consistent with previous observation in transgenic mice with overexpression of Ang-2 in the retina which exhibit increased pre- and intra-retinal angiogenesis [3], the data indicate the cooperative role of Ang-2 with VEGF in vessel formation of both, pre- and intra-retinal vessels.

We found that Ang-2 is predominantly expressed at the leading edges of intra-retinal sprouts in the OIR model. Our data also support previous studies showing that Ang-2 is expressed at the front of invading vascular sprouts during vascular remodeling in the ovary [25]. In the OIR model, localization of Ang-2 in pre-retinal neovascularizations and intra-retinal capillaries has been demonstrated [7]. Expression of Ang-2 in central artery in the eye and in mesenteric arteries has been documented in a study by Gale et al [23]. Our study demonstrates that Ang-2 is localized in pre-retinal neovascularizations, in arterioles, on the sprouts of venules and capillaries towards the retinal deep layer. Taken together, these studies indicate the particular significance of Ang-2 at the active angiogenic front.

MMPs are activated in the OIR model and localized in pre-retinal neovascularizations [18]. We found that MMPs are dominantly active in arterioles and on the vascular sprouts, similar to where Ang-2 is expressed. The general role of combined Ang-2/MMPs activity is further supported by data from invasive tumors. Elevated Ang-2 in tumor cells caused activation of MMPs leading to increased invasiveness of malignant tumors. High levels of Ang-2 and MMPs are specifically found in invasive areas of tumor sites [20]. A third example for the collaboration of Ang-2 and MMPs in playing a role in tissue remodeling is represented by sprouting angiogenesis in adult skeletal muscle. Here, up-regulation of angiogenic factors, such as VEGF and Ang-2, coincides with MMP activity in the extracellular matrix [26]. Similarly, strong intra-retinal activation of MMPs in arterioles, on the vascular sprouts indicates that Ang-2 with adequate activated MMPs is needed not only for pre-retinal neovascularization, but also strongly in assembling the intra-retinal vascular network. Requirement of Ang-2 and activated MMPs in arterioles and on the sprouts is likely dependent on high angiogenic and degradative demand because of their special existing matrix components.

In the OIR model, MMP2 and MMP9 are upregulated [13]. More decreased neovascularizations by MMP2 selective inhibitor and in MMP2 deficient mice than by MMP2+MMP9-selective inhibitor and in MMP9 deficient mice suggest that MMP2 is likely a more dominant mediator than MMP9 in formation of retinal neovascularization in this model [27]. However, the study by Das et al. demonstrated that angiopoietin-moderated retinal neovascularization may be in part through the inhibition of MMP9 expression [18]. Our data on specificity of MMPs using loss of function of Ang-2 support the observation of Das and colleagues, indicating

that Ang-2 exerts its effect through activation of MMP9. However, the molecular mechanism(s) by which Ang-2 mediates MMP activation is unknown at present and requires further investigations.

In conclusion, our study provides evidences that VEGF-mediated angiogenesis under hypoxia is modulated by Ang-2. Ang-2 and activated MMPs on the fronts of angiogenesis play a critical role in proliferative retinopathy.

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